Novel silencing of alcohol dehydrogenase in *Schizosaccharomyces pombe*

A Senior Honors Thesis

Presented in Partial Fulfillment of the Requirements for graduation *with research distinction in Molecular Genetics* in the undergraduate colleges of The Ohio State University

by
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Abstract

Non-protein coding RNAs (ncRNAs) have emerged as a significant factor in gene regulation. In this thesis, we have studied the expression profile of the *Schizosaccharomyces pombe* gene alcohol dehydrogenase 1 (*adh1+*). Adh1 is an abundant zinc binding protein that is repressed in low zinc in order to conserve zinc for other cellular processes. We have found that *adh1+* is repressed in response to severe zinc deficiency, and the repression correlates to expression of an antisense transcript larger than the gene. When we expressed *adh1+* constitutively from the *nmt1+* promoter, we found that this did not affect the zinc-mediated regulation of *adh1+*. In addition, artificial repression of the sense transcript did not prevent zinc-mediated regulation of the antisense transcript. The regulation of this locus by zinc was studied over time, and it was determined that the sense transcript is fully repressed by four hours, but it is not yet known whether the sense and antisense transcripts are mutually exclusive.

Acknowledgements

This thesis is greatly indebted to Doctor Amanda Bird, who is well versed in zinc metabolism. She provided the direction for the experiment and pointed out many publications significant to the work to be done. She was always approachable and willing to banter back-and-forth about mechanisms.

Carter Mason was also a great help to the experiment. His diligent work was crucial to the smooth operation of the laboratory.

Contents

Abbreviations

[S]	sense transcript of a locus
[AS]	antisense transcript of a gene locus
|¯>	promoter sequence of a gene
HybMap	RNA-DNA hybridization mapping
miRNA	micro RNA
ncRNA	non protein-coding RNA
RNAi	RNA interference
siRNA	silencing RNA

List of Diagrams and Figures

Introduction

Non-protein coding RNAs (ncRNAs) are a matter of growing significance in eukaryotic biology. In addition to mRNA, rRNA, and tRNA, RNAs are also involved in mediating splicing (snRNA), modification of rRNA (snoRNA), post-transcriptional silencing (siRNA and miRNA), and RNA editing (gRNA). Transcriptome profiling has recently revealed that the greater fraction of most genomes is transcribed (Cheng et al., 2005; Dutrow et al., 2008; Xu et al., 2009). While it has been traditionally thought that the large majority of these transcripts were simply junk RNA, it is now clear that many of these intergenic transcripts have vital cellular functions, suggesting that there are many new roles of RNA yet to be discovered (Carninci et al., 2008; Mercer et al., 2009; Shamovsky et al., 2006; Shamovsky and Nudler, 2009).

One of the major classes of ncRNAs is antisense transcripts, and some of the best studied of those are from the budding yeast *Saccharomyces cerevisiae*. Hongay et al. demonstrated that antisense transcription controls the expression of *IME4*, one of the *S. cerevisiae* genes responsible for initiating meiosis in MAT a/α diploids (Hongay et al., 2006). Two transcripts are synthesized at the *IME4* locus (Diagram 1). One transcript is the mRNA. A second, longer

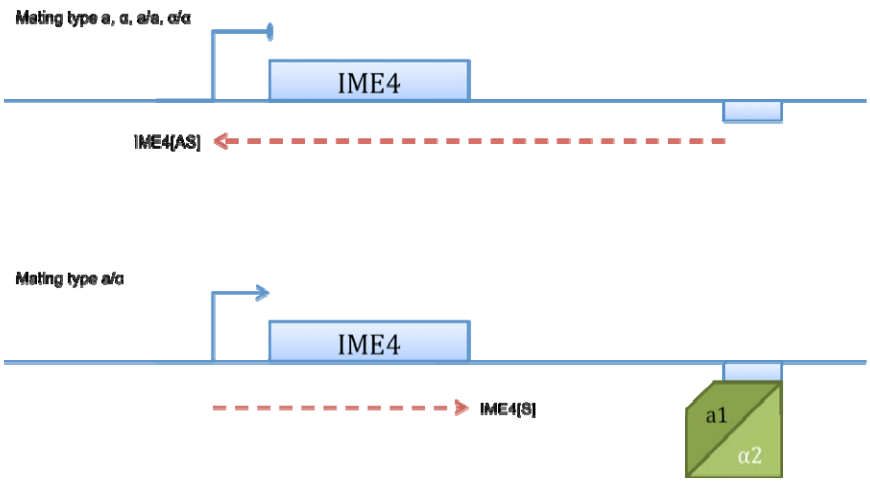


Diagram 1: regulation of *IME4*

antisense transcript is transcribed from the opposite strand. The sense transcript is only produced in diploid MAT a/α cells, while the antisense transcript is only expressed in haploid cells. Regulation at the *IME4* locus is accomplished by the antisense transcript. Synthesis of the antisense transcript inhibits sense transcription by physically interfering with the ability of transcription factors to bind to the sense promoter. MAT a/α cells suppress the antisense transcript through the activity of the a1/α2 heterodimer. Without the interference of the antisense transcript, transcription factors are free to bind to the sense strand and induce expression of *IME4* (Hongay et al., 2006).

Camblong et al. have also demonstrated regulation by antisense transcript at the *PHO84* locus of *S. cerevisiae* (Camblong et al., 2007). At the *PHO84* locus, deletion of the exosome component Rrp6 induces stabilization of the *PHO84* antisense transcript (PHO84[AS]). This stabilization in turn reduces PHO84[S] levels. Stabilization of PHO84[AS] is linked to the recruitment of histone deacetylases, which prevent sense strand expression at this locus (Camblong et al., 2007). Thus, there are a variety of mechanisms by which antisense transcription might regulate gene expression.

This thesis concerns the activity of the *S. pombe* gene alcohol dehydrogenase 1 (*adh1+*) and its regulation in response to cellular zinc status by antisense transcription. Adh1 is an abundant cytosolic zinc-binding protein. In normal growth media, it is estimated that up to 10% of cellular zinc is bound to Adh1 (Eide, 2003). In *S. pombe*, *adh1+* is the most highly repressed gene in response to zinc deficiency. Reducing levels of Adh1 in low zinc is likely a means of conserving zinc for use by more essential zinc binding proteins.

There is precedent for zinc-mediated regulation of *ADH1* in *S. cerevisiae (Bird et al., 2006; Wu et al., 2008)*. In this yeast species, *ADH1* expression is regulated by three transcription factors, Zap1, Rap1, and Gcr1 (Diagram 2). Under normal conditions, the Rap1/Gcr1 heterodimer binds upstream of *ADH1* and activates its transcription. When starved for zinc, the cell produces the transcription factor Zap1. Zap1 binds to the *ADH1* promoter upstream of the Rap1/Gcr1 binding site and promotes the transcription of the ncRNA *ZRR1*. Thus, when *ZRR1* is synthesized, it displaces Rap1/Gcr1 and prevents transcription of *ADH1*. In addition to repressing *ADH1* gene expression, Zap1 also activates the expression of a different alcohol dehydrogenase gene, *ADH4*. Adh4 binds an iron cofactor instead of zinc. This allows *S. cerevisiae* to tailor alcohol dehydrogenase gene expression to metal availability (Bird et al., 2006; Wu et al., 2008).

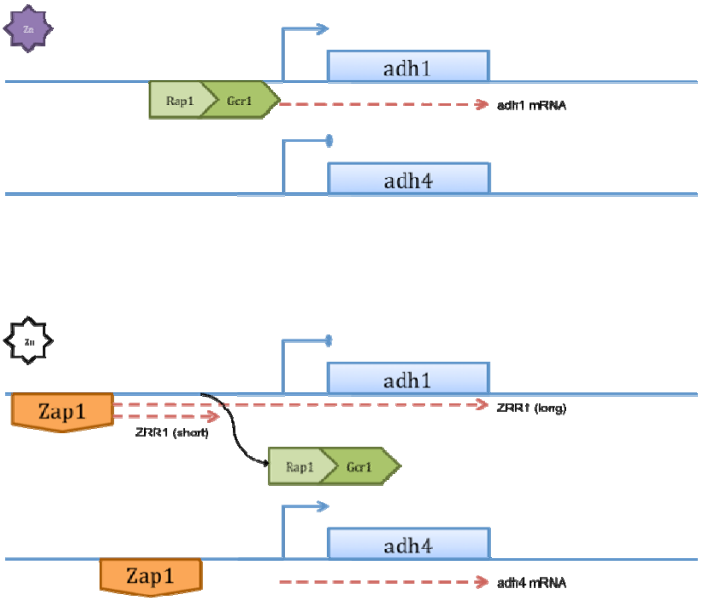


Diagram 2: regulation of *ADH1*

In *S. pombe*, an antisense transcript is generated at the *adh1+* loci. Unlike *S. cerevisiae*, *S. pombe* contains the cellular machinery for RNA interference (Moazed, 2009). RNA interference (RNAi) constitutes a significant portion of gene regulation. This mechanism was first identified in *C. elegans*, and the resulting epigenetic changes were found to be heritable

(Lee et al., 1993). Interfering RNAs are produced through the activities of Drosha and Dicer (Rana, 2007; Wu and Belasco, 2008). When the original transcript (called the primary micro RNA or pri-miRNA) is synthesized from the genome, it contains inverted repeats that allow it to fold into a stem-loop (Diagram 3). Drosha cleaves the stem-loop from trailing single-stranded RNA (ssRNA), leaving the mostly double-stranded (dsRNA) stem-loop (now called the pre-miRNA) for later processing. Dicer recognizes this dsRNA structure and digests it into fragments approximately 20 nucleotides long, mature miRNA. One strand of these fragments is then collected by the RNA-induced silencing complex (RISC), specifically the catalytic subunit Argonaute. Argonaute binds to mRNAs complementary to its miRNA load. Binding of Argonaute can lead to mRNA cleavage or translational repression, thereby inhibiting the expression of that gene (Rana, 2007; Wu and Belasco, 2008).

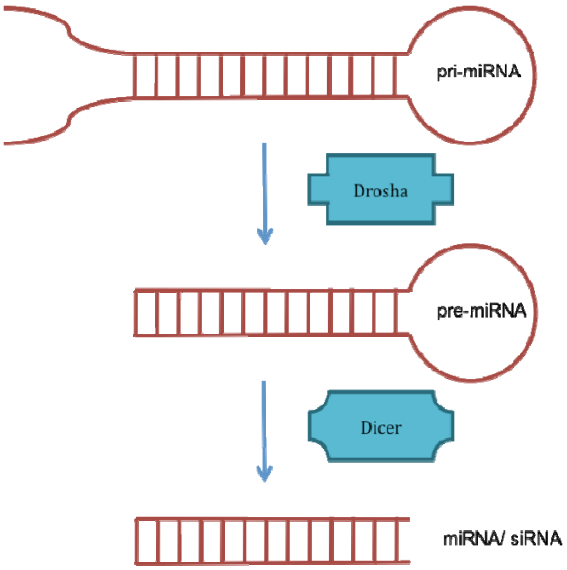


Diagram 3: RNAi pathway

Starting with Dicer, this pathway can also process dsRNA produced in any other fashion. RNAi fragments resulting from such RNAs are called siRNA. *S. pombe* lacks Drosha, and so can not process miRNA, but it is still capable of producing siRNA (Moazed, 2009). In *S. pombe,* siRNAs act at a transcriptional level and accomplish silencing by chromatin modification at the site of nascent RNA production (observed at the centromeres, the telomeres, and the mating type region). In the particular cases of centromeres and the mating type locus, *S. pombe* makes use of RNA-induced transcriptional silencing (RITS). The RITS complex contains the *S. pombe*

Argonaute protein Ago1, as well as Chp1 (a chromodomain protein) and Tas3 (which bridges the former two). Like RISC, the RITS complex seeks RNA complementary to the RNA carried by its Argonaute, but its purpose is to find the DNA associated with that transcript and initiate heterochromatin formation at that site. This is accomplished by methylation of lysine 9 of histone H3 (Moazed, 2009).

In light of the existing research, the synthesis of an antisense transcript complementary to mRNA admits the possibility of several silencing mechanisms in *S. pombe*. In this thesis, we sought to determine the precise method used at the *adh1+* locus. We have determined that full repression of the adh1[S] is accomplished by 4 hours of growth in zinc-limiting media. In addition, the zinc-mediated regulation of *adh1+* is not dependant upon the natural *adh1+* promoter.

Materials and Methods

Genomic DNA extraction

Protocol provided by Dr. Jian-Qiu Wu of the Department of Molecular Genetics at The Ohio State University.

4 mL of cell culture was grown to stationary phase in EMM. The culture was spun down and the supernatant decanted. The cells are resuspended in 0.5 mL dH_2O. Following centrifugation, the supernatant was removed, leaving behind 100 µL of residual dH_2O. The cells were resuspended in the residual dH_2O before the addition of 200 µL of Blue Buffer, 200 µL of phenol-chloroform-isoamyl alcohol (25:24:1), and 0.3 g acid-washed glass beads. The solution was vortexed for 3 min. to lyse the cells before centrifugation at 12000 rpm for 6 min. 160 µL of the top layer of solution was removed and DNA precipitated by the addition of 1 mL of absolute ethanol. The solution is centrifuged for 2 min and the supernatant discarded. The pellet was dried and resuspended in 40 µL of Qiagen Elution Buffer. Genomic DNA was stored at –20°C

RNA purification

Cells were grown to stationary phase in a 50 mL culture of EMM. The supernatant was removed by decanting after centrifugation and by pipetting after a second centrifugation. The cells were resuspended in 650 µL TES solution with 650 µL acidic phenol (Phenol/Chloroform/Isoamyl alcohol, pH 4.3), vortexed briefly to mix, and incubated at 65°C for 1 hr to lyse the cells. The solution was placed on ice for 3 min before centrifugation at 12000 rpm for 5 min at room temperature. The upper layer is removed and added to 500 µL acidic phenol, vortexed to mix, and centrifuged (12000 rpm, 5 min., room temperature). The upper

layer was removed and added to 400 μL chloroform, vortexed to mix, and centrifuged again (12000 rpm, 5 min., room temperature). The upper layer was then added to 50 μL 3M sodium acetate and 900 μL absolute ethanol and stored overnight at –20°C to precipitate RNA. The sample was centrifuged again (12000 rpm, 10 min., 4°C) and the supernatant decanted. The pellet was washed with 70% ethanol and air dried for 30 min at 37°C. The pellet was suspended in dH2O at 65°C. RNA was separated on 1.5% formaldehyde agarose gels and transferred to a nitrocellulose membrane by standard procedures (Sambrook and Russell, 2001).

Transformation

Cells were grown to stationary phase in 50 mL of yeast extract media. The culture was centrifuged and the supernatant discarded. The pellet was washed in 5 mL lithium acetate, centrifuged again, and the supernatant discarded again. The pellet was then resuspended in residual lithium acetate. 60 μL of cell solution was incubated with 7 μL of the kanamycin insert at 31°C for 20 min. The cells were then treated with 7 μL of denatured salmon sperm DNA and 320 μL lithium acetate with 40% PEG and incubated at 31°C for another 20 min. The cells were then exposed to heat shock at 42°C for 15 min. and centrifuged. The supernatant was discarded, and the cells were resuspended in 150 μL of yeast extract media. The cells were allowed to grow on yeast extract plates, after which they were replica plated onto yeast extract with G418 to select for kanamycin resistance (Bahler et al., 1998).

Cell growth conditions

Cells were suspended in 5 mL Edinburgh minimal media and grown for 24 hrs at 31$^{\circ C}$. The culture was added to 30 mL of additional media and grown for a further 24 hrs. The

experimental cultures were then established by taking 5 mL aliquots from this culture and adding it to 45 mL of media. Zinc deficient cultures were made by adding EDTA to a final concentration of 100 μM. Thiamine replete cultures were made according to the instructions of the Forsburg laboratory. Cultures were then allowed to grow for a set time, after which they were centrifuged. The supernatant was decanted, and the cells were stored at -80°C for later processing.

Growth media

Media used for cell cultures were Edinburgh Minimal Media and yeast extract media, prepared as per the directions of the Forsburg laboratory. All cultures included supplements of adenine, histadine, leucine, and uracil. Instructions may be found at

http://www-rcf.usc.edu/~forsburg/media.html

Experimental strains

Experiments were performed on the JW81 strain of *S. pombe* (mat h-, ade6-M210, leu1-32, ura4-D18). The nmt1|⁻⁻>adh1 transformant was made from the same background and includes three HA tags at the *adh1+* locus.

Results

Preliminary array analysis

In preliminary studies by the Bird lab, transcription of the entire *S. pombe* genome and how it changes between zinc deficiency and zinc excess was analyzed through the HybMap system (Dutrow et al., 2008). Hyb Map, or DNA-RNA hybridization mapping is a new array technology that allows study of RNA without the need for amplification. The tiling array used in the *S. pombe* HybMap contains 458,566 60-mer DNA oligonucleotides as probes. Together the probes account for the entire *S. pombe* genome with a 5 base overlap between neighboring probes and a genome resolution of 50 bases. In the HybMap protocol, total RNA is hybridized to the array, and successful DNA-RNA hybrids are detected using the S9.6 primary antibody (Diagram 4). The primary antibody recognizes DNA-RNA hybrids of 15 bp in a sequence-unspecific manner and has greatly reduced affinity for a 1 bp mismatch. Following incubation with the primary antibody, the array is incubated with a secondary antibody conjugated to the Cy3 fluorophore. This method identifies all transcripts in the cell without amplification and without regard for polyadenylation status. This array system is useful for identifying ncRNAs since ncRNAs need not be polyadenylated (Dutrow et al., 2008).

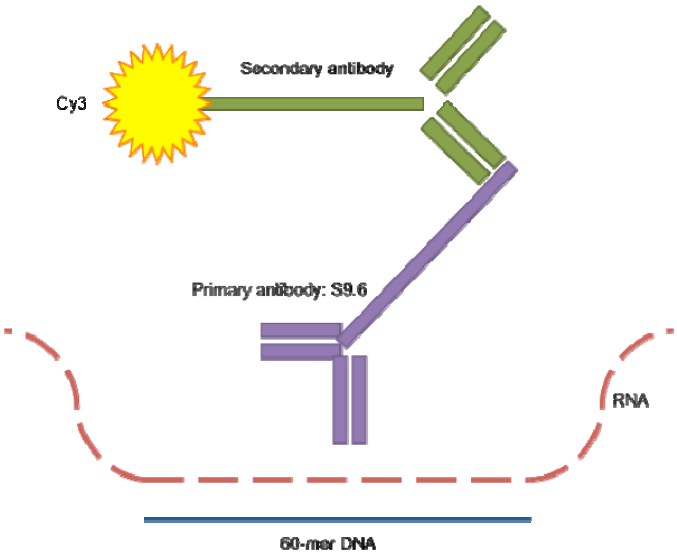


Diagram 4: a transcript identified by HybMap

To identify ncRNAs that may have regulatory roles, three extragenic loci were chosen that show strong repression in response to zinc deficiency (Figures 1, 2, and 3). For the purposes of this study, these ncRNAs are designated ncRaz1, ncRaz2, and ncRaz3 (*n*on-protein *c*oding RNA *r*epressed in the *a*bsence of *z*inc). To determine the function of these ncRNAs, wild type cells were transformed by homologous recombination with a kanamycin cassette to have disruptive insertions at one of those loci (Diagram 5). Individual knockout strains of ΔncRaz2::kan and ΔncRaz3::kan were successfully produced. These transformants were verified by diagnostic PCR. In diagnostic PCR, two primers are used: one complementary to the sequence of the insert and one complementary to the native sequence immediately outside the insert (Diagram 6). Untransformed DNA lacks one of the binding sites. Thus, only transformed cells, in which the kanamycin cassette is integrated into the correct place in the genome, will produce an amplification product. The knockout strains were further analyzed by Northern analysis to confirm the transformation. Total RNA was purified from both wild type and deletion strains grown in both zinc-replete and zinc-starved conditions. The resulting blots were probed for *cam1*+ (calmodulin 1) transcripts as a control, for *cam1*+ is not sensitive to cellular

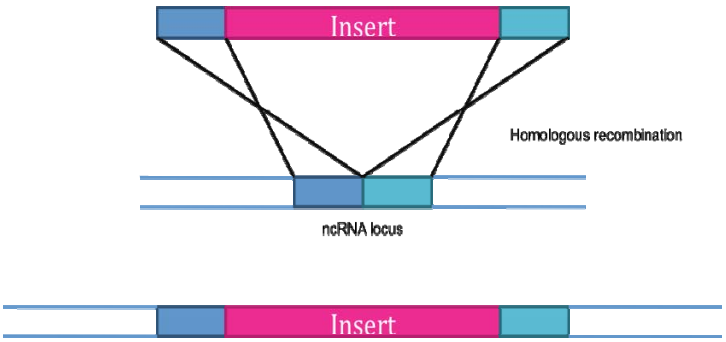


Diagram 5: insertion by homologous recombination

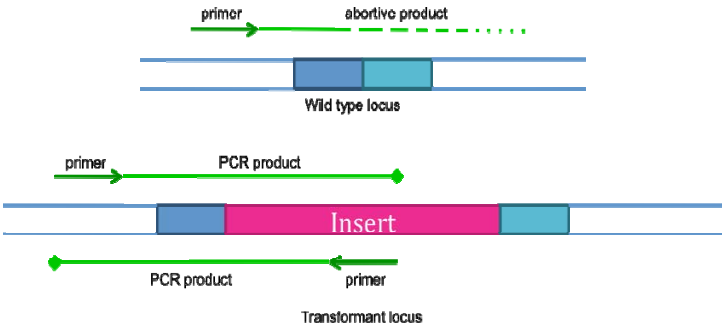


Diagram 6: diagnostic PCR

zinc status. The *cam1+* probe was positive, proving that the Northern blot was successful; however, probes against ncRaz2 and ncRaz3 failed to detect transcripts specific to those ncRNAs (data not shown). At this point, the focus of the experiment turned to *adh1+* in deference to its more favorable properties.

Zinc-mediated control of the *adh1+* locus

The *adh1+* locus exhibits the greatest difference in transcription in the entire *S. pombe* genome between zinc-replete and zinc-starved conditions. *adh1*[S] is the most repressed mRNA and *adh1*[AS] the most induced ncRNA measured by the array. In order to confirm the zinc-mediated regulation of the *adh1+* locus, wild type cultures of *S. pombe* were grown up to exponential phase under both zinc-replete and zinc-starved conditions. RNA was purified from the cells and separated on a formaldehyde gel. The gel was subjected to a Northern blot, and the resulting nylon membrane was probed for *adh1*[S], *adh1*[AS], *adh4+*, and *cam1+* transcripts (figure 5). Calmodulin transcription is not affected by zinc deficiency, and so it serves as the loading control. Adh4 is another alcohol dehydrogenase which binds iron instead of zinc. In both *S. cerevisiae* and *S. pombe*, *adh4+* is specifically induced in response to zinc starvation. It therefore serves as a control for zinc deficiency (Dainty et al., 2008). The data confirmed the results of the HybMap transcriptome: *adh1*[S] is induced in zinc-replete conditions and repressed in zinc-starved conditions, and *adh1*[AS] displays reciprocal activity.

Alternate transcriptional control of the *adh1+* locus

Repression at the *adh1+* locus could be mediated by a number of mechanisms, including transcriptional interference and chromatin remodeling as mentioned previously. To determine

the relevance of the *adh1*+ promoter to the zinc-mediated regulation of *adh1*[S] and *adh1*[AS], wild type *S. pombe* cells were transformed to place *adh1*+ under the control of the promoter of *nmt1*+. The *nmt1*+ gene is part of the thiamine biosynthesis pathway. It is well characterized for its strong response to thiamine. The promoter is repressible in thiamine and is used experimentally to exert alternate transcriptional control on target genes (Basi et al., 1993; McQuire and Young, 2006). To generate a strain containing *adh1*+ under the *nmt1*+ promoter, wild-type cells were transformed with a cassette containing the kanamycin resistance gene and the *nmt1*+ promoter. Primers were designed to amplify the kanamycin cassette, such that homologous recombination would place the *nmt1*+ promoter directly upstream of the *adh1*+ coding region. This strain is designated nmt1|¯¯>adh1.

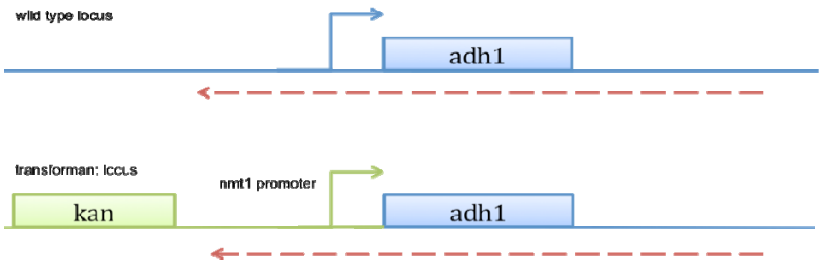


Diagram 7: nmt1|¯¯>adh1 construct

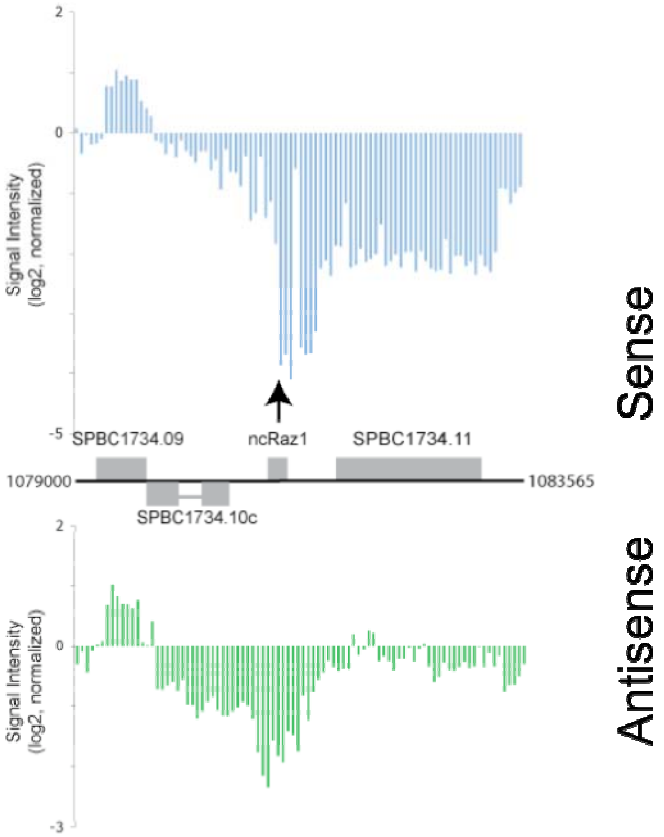
nmt1|¯¯>adh1 cells were grown to exponential phase in parallel with wild type cells under both zinc-replete and zinc-starved conditions as well as with and without thiamine. RNA was extracted from the cells, separated on a formaldehyde gel, blotted, and probed for *cam1*+ (loading control), *adh4*+ (zinc control), *adh1*[S], and *adh1*[AS] as above. The nmt1|¯¯>adh1 transformants and the wild type cells show identical transcriptional activity for *adh1*[S] in the absence of thiamine (figure 5). Thiamine has no effect on *adh1*[S] in the wild type, but in the nmt1|¯¯>adh1 strain, it has the intended effect of silencing *adh1*[S]. *adh1*[AS] is larger than *adh1*+ itself, so by replacing adh1|¯¯> with nmt1|¯¯>, we have also replaced approximately 1 kb of

the 3' end of *adh1*[AS]. This is observable in the Northern blot in that *adh1*[AS] runs as a shorter transcript. Aside from this decreased length, however, *adh1*[AS] transcription is identical between the wild type and nmt1|¯>adh1 strains in the absence of thiamine. In the presence of thiamine, *adh1*[AS] shows a stronger reaction in the transformant than in the wild type.

Silencing of the *adh1*+ locus over time

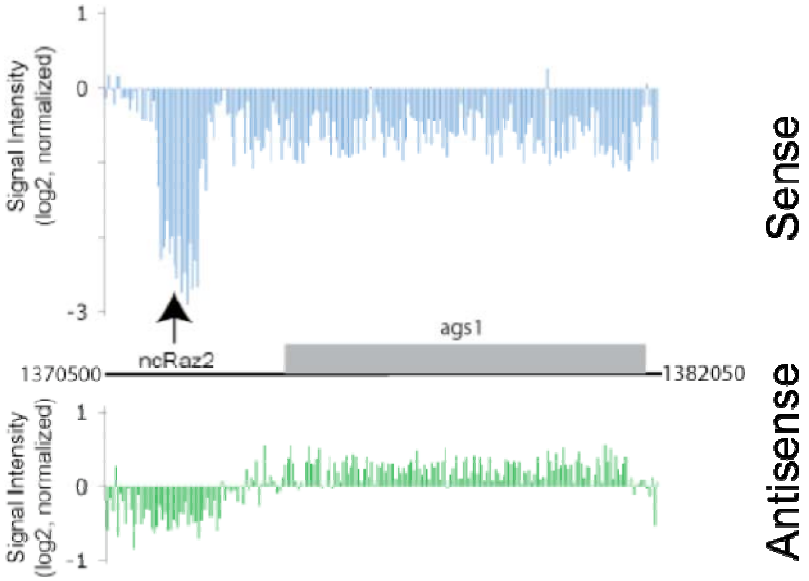
Since the *adh1*+ locus produces two complementary transcripts, *adh1*+ may be regulated through RNAi. As such, it is necessary to determine if *adh1*[S] and *adh1*[AS] are ever transcribed concurrently. To determine this, wild type cells were grown to exponential phase in zinc-replete conditions and then divided into zinc-replete and zinc-starved cultures. Samples of each were collected at 30 min., 90 min., and 240 min. RNA was extracted from the samples, separated on a formaldehyde gel, blotted, and probed for *cam1*+ (loading control), *adh4*+ (zinc control), *adh1*[S], and *adh1*[AS] (figure 6) in the method described above. The zinc-starved cultures exhibit decreased *adh1*[S] activity over time, with no *adh1*[S] observed after 240 min. *adh1*[AS] displays opposite activity: no activity was observed by 90 min., but the response was strong after 240 min. In contrast, *adh4*+ was already active by 30 min.

Figure 1: Transcriptome profile of the ncRaz1 locus



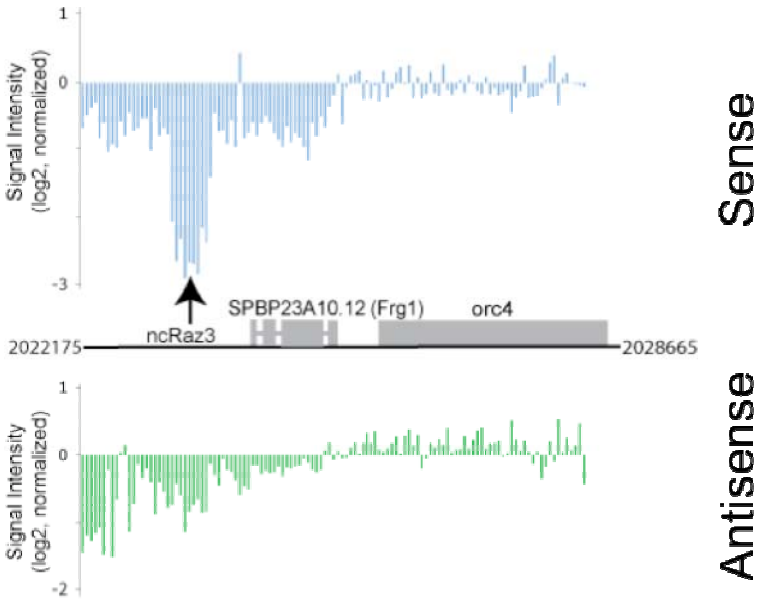
This graph was generated from HybMap data of the ncRaz1 locus. ncRaz1 is a small sequence (~150 b) that shows approximately 16-fold repression in zinc-starved conditions.

Figure 2: Transcriptome profile of the ncRaz2 locus



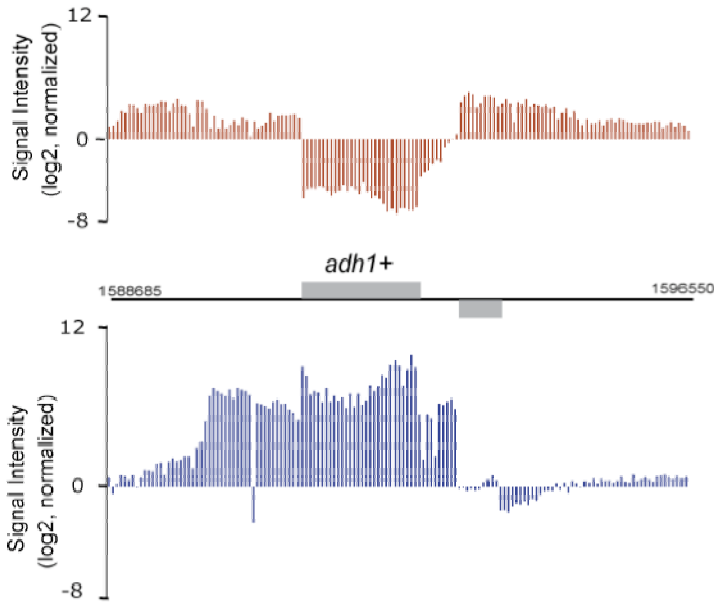
This graph was generated from HybMap data of the ncRaz2 locus. ncRaz2 is upstream of the alpha-1,4-glucan synthase gene (*ags1+*) and exhibits approximately 6-fold repression in the absence of zinc.

Figure 3: Transcriptome profile of the ncRaz3 locus



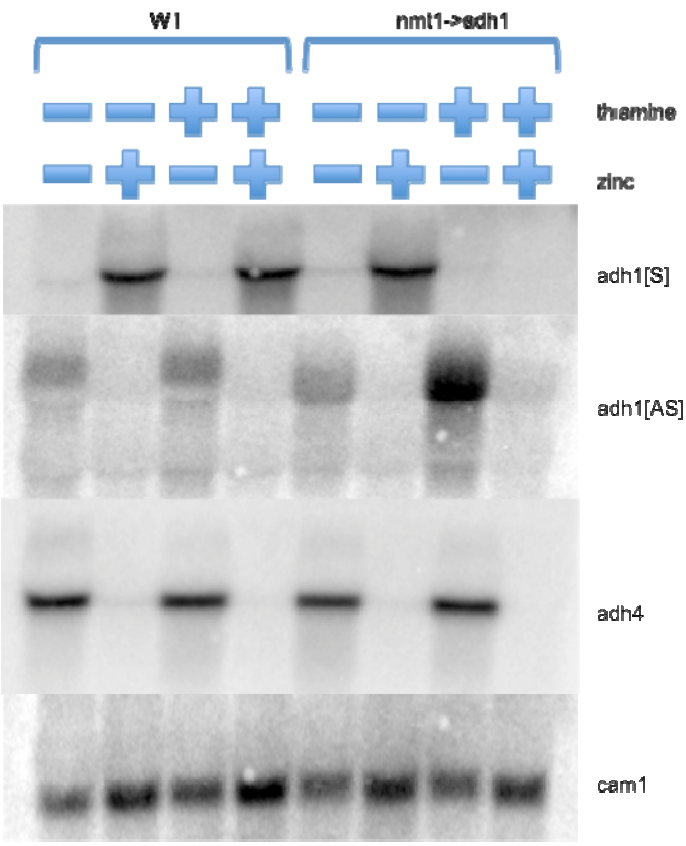
This graph was generated from HybMap data of the ncRaz3 locus. ncRaz3 exhibits approximately 7-fold repression in the absence of zinc and is upstream of *frg1+*, a homolog to a human gene linked to muscular dystrophy.

Figure 4: Transcriptome profile of the *adh1+* locus



This graph was generated from HybMap data of the *adh1+* locus of *S. pombe*. The data show a strong decrease in expression sharply delineated at the beginning of the *adh1+* ORF and containing the entire gene. On the opposite strand is an ORF of unknown function: SPCC13B11.02c. At the end of this ORF is the beginning of a large region of increased transcription. The transcript is more than 3 kb long, spanning the entire length of *adh1+* and including sequence both upstream and downstream as well.

Figure 5: Northern blot of nmt1|¯>adh1



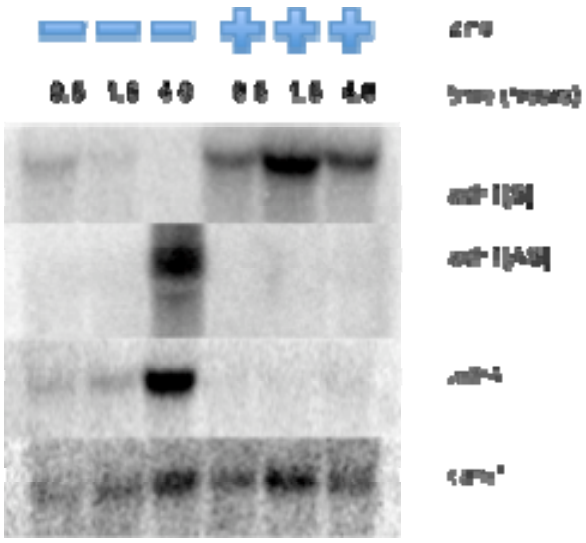
This series of photos shows the Northern blot from the nmt1|¯>adh1 experiment after exposure to probes against *adh1*[S], *adh1*[AS], *adh4*+, and *cam1*+. The transcriptome profile indicates that calmodulin transcription is not affected by cellular zinc status. It is also not influenced by thiamine. It serves as a control for this experiment for loading concentration. This picture shows that the concentration of RNA is roughly constant between lanes. The transcriptome profile also shows that *adh4*+ is repressed in zinc-replete conditions and induced in zinc-starved conditions. This picture confirms that result. In addition, *adh4*+ transcription has not been affected by the integration of nmt1|¯> in front of *adh1*+, nor was it affected by thiamine. The blot thus demonstrates that the intended cultures have indeed been starved for zinc.

This picture confirms the data from the transcriptome profile, i.e. that *adh1*[S] is induced in zinc-replete conditions and repressed in zinc-starved conditions. In the absence of thiamine, the nmt1|¯>adh1 displays the same transcription profile as the wild type. Thiamine shows no effect

on *adh1*[S] in the wild type; however, the nmt1|¯>adh1 transformant exhibits repression of *adh1*[S] in the presence of thiamine.

As expected from the transcriptome profile, *adh1*[AS] is repressed in zinc-replete conditions and induced in zinc-starved conditions in the wild type. The nmt1|¯>adh1 transformant demonstrates the same binary expression profile as the wild type irrespective of thiamine in the environment. However, *adh1*[AS] runs longer on the gel in the nmt1|¯>adh1 strain, indicating that the transcript is shorter.

Figure 6: Northern blot of zinc starvation over time



This series of photos shows the Northern blot for zinc starvation over time after exposure to probes against *adh1*[S], *adh1*[AS], *adh4*+ and *cam1*+. As shown in the bottom picture, the activity of calmodulin (the loading control) is not uniform between lanes. This suggests that lanes 3 and 5 contain greater quantities of RNA than the other lanes. Due to variation in RNA, no statement can be asserted about the relative level of *adh4*+ activity in the blot save to say that *adh4*+ does appear in the zinc-starved lanes (1, 2, and 3) and does not appear in the zinc-replete lanes, demonstrating the correct zinc status in the cultures. Moreover, the cell has already reacted to the lack of zinc by 30 min.

The difference in *adh1*[S] probe strength in lanes 4, 5, and 6 confirms the result from the loading control that lane 5 contains more RNA. However, despite the greater content of RNA in lane 3, no *adh1*[S] activity is observed after 240 min. of zinc starvation.

Figure 7: Microscopy image of *S. pombe*

Image provided by Dr. Jian-Qiu Wu of the Department of Molecular Genetics at The Ohio State University.

Discussion

From the combined data of the HybMap profile and Northern analysis, we have found that *adh1+* expression is regulated by cellular zinc status and that the mechanism of repression likely involves antisense transcription. In the nmt1|⁻⁻>adh1 experiment, the wild type and transformant cultures showed identical *adh1*[S] expression, so the natural *adh1+* promoter is not necessary for the zinc-mediated regulation of *adh1+*. These data would suggest that the repression at *adh1+* promoter does not involve the recruitment of a transcriptional repressor. Furthermore, since *adh1*[AS] activity increases when *adh1*[S] is artificially repressed, it is possible that *adh1*[AS] regulates *adh1*[S]. To test this, we intend to study two mutant strains. The first is a deletion strain for the unknown ORF (*SPCC13B11.02c*) upstream of *adh1*[AS]. *SPCC13B11.02c* lies directly in the *adh1*[AS] promoter. We intend to determine whether this ORF influences the transcription of *adh1*[AS]. The second mutant for study is an nmt1|⁻⁻>adh1[AS] transformant. This would allow us direct control of *adh1*[AS] transcription through thiamine. Thus we may determine whether *adh1*[AS] is directly responsible for *adh1*[S] regulation.

We also observe that the insertion of nmt1|⁻⁻> replaced approximately 1 kb of the 3' end of *adh1*[AS], but, while this resulted in a shortened *adh1*[AS], curiously, this had no detectable effect on the regulation of *adh1+*. Therefore, neither the 3' sequence nor the precise length of *adh1*[AS] is necessary for zinc-mediated silencing. Furthermore, the thiamine mediated silencing of *adh1*[S] shows that *adh1*[S] activity is not required for *adh1*[AS] expression.

Indeed, *adh1*[S] appears to have an antagonistic effect on *adh1*[AS] that is reduced by artificial silencing.

The goal of the time-dependent zinc starvation experiments was to investigate whether the sense and antisense strands are ever concurrently synthesized or if they are mutually exclusive. The difference is significant because if ever they both exist in the cell at the same time, they can hybridize (forming a dsRNA) and enter the RNAi pathway. Other experiments on RNAi have isolated fragments of both *adh1*[S] and *adh1*[AS] from Argonaute (Buhler et al., 2008). Unfortunately, from the pilot studies, it is not yet clear whether this is significant. Future studies would entail repeating this experiment with additional time points for better resolution. There are a number of mechanisms described in the literature that may act on the *adh1+* locus. Much work remains to distinguish between them.

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Vita

Andrew Patrick began his interest in genetics with *The Cartoon Guide to Genetics* by Larry Gonick and Mark Wheelis. From reading that book, he moved on to intern under Dr. Kathleen Barnes of Johns Hopkins University. He presented his research into asthma in humans as part of the requirements for graduation from Long Reach High School in the Biotechnology cluster of the Technology Magnet program.

Andrew is currently a fourth year student in the undergraduate program of the Department of Molecular Genetics at The Ohio State University. He plays the viola and enjoys classical music, chess, and reading.